Antagonistic activity of a novel chitosan-selenium nanoflower against common aquaculture pathogen Aeromonas caviae

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Abstract

Aquatic pathogens contribute the most severe economic loss in fishes. Nanoparticles are being developed as potent antimicrobial agents against various pathogens. This study involved the synthesis and characterization of a novel chitosan-selenium nanoower employing multiple spectroscopic and microscopic approach. The UV-vis spectra obtained at 265 nm indicated the formation of the Chitosan-selenium nanoower. The Particle size analysis revealed the size of the nanoowers to be 186.3 nm. The Transmission Electron Micrographs revealed a unique nanoower like morphology. XRD spectrum revealed amorphous nature and the Raman spectrum showed characteristic resonance peak at 254 cm\(^{-1}\) was a characteristic absorption band for monoclinic Se and α-Se. Cytotoxicity analysis of the synthesized nanoowers against isolated fish pathogen \(A.\ caviae\) showed increasing toxicity in a dose dependent manner. The DCFDA assay was conducted for estimating increase in ROS production and the highest percentage increase in ROS was observed at 1000 µg/mL. The lipid peroxidation assay was performed by quantification of lipid oxidation product Malondialdehyde (MDA). The highest percentage lipid peroxidation was found to occur at a dose of 500 µg/mL. As a result, the synthesised chitosan-selenium nanoowers can be exploited as a promising antibacterial treatment against the fish pathogen \(A.\ caviae\).

1. Introduction

The main barrier to development of sustainable aquaculture is the rise of several diseases. One of the main causes of the majority of fish fatalities worldwide is bacterial infections in farmed fish (Rathore et al. 2021). Around the world, \(Aeromonas\ species\) is frequently found in aquatic environments and is the primary cause of serious fish infections (Baldissera et al. 2018a). With a 1-2% prevalence compared to other \(Aeromonas\ species,\ \(Aeromonas\ caviae\) has attracted attention as a significant fish pathogen (Vega-Sánchez et al. 2014). A large variety of economically significant fish, including \(Clarias\ gariepinus\) (African catfish), \(Colossoma\ macropomum\) (tambaqui) \(Onchorhynchus\ mykiss\) (rainbow trout) \(Clarias\ batrachus\) (Indian walking catfish) and \(Rhamdia\ quelen\) (silver catfish) (Thomas et al. 2013; Vega-Sánchez et al. 2014; Marques et al. 2016; Sarjito et al. 2017; Baldissera et al. 2018b), are susceptible to infection by this gram-negative bacterium. This bacterium has been recognized in fish as one of the most common causes of hemorrhagic septicemia in fish. It affects fish and is associated with hepatosplenomegaly, ulcerative skin lesions (Thomas et al. 2013), and disruption of the hepatic energy metabolism (Baldissera et al. 2018b). There are several reports associating significant fish mortality to the presence of pathogenic \(A.\ caviae\) (Munro et al. 1993; Ringo and Vadstein 1998). High percentages of \(A.\ caviae\) were linked to low fish larval survival rates. In the intestines of turbot fish larvae with high \(A.\ caviae\) infection, 100% mortality was observed (Ringo and Vadstein 1998). There are numerous examples of \(A.\ caviae\) being isolated from other fish, including mullet, carp, catfish, goldfish, and tilapia (Sugita et al. 1994). This not only causes mass mortality in fishes but also brings about significant economic losses for the aquaculture industries.
Numerous innovative nanomaterials have drawn a lot of attention lately because of their enormous potential in the fields of engineering, agriculture, food safety, and biological sciences. The overuse of antibiotics have led to the evolution of very many antibiotic resistant pathogens (Schmidt et al. 2000). To overcome the effects of antibiotics and antibiotic-resistant pathogens, various innovative nanomaterials have been used in fields of aquaculture. Among these, selenium (Se) nanoparticles are demonstrating promise as a nanomaterial with a diverse range of potential uses. In various living creatures, including humans, animals, bacteria, and archaea, the element Se is recognized as an essential micronutrient. For the health of both humans and animals, Se is an essential trace element. The daily dietary need for Se in humans is 55 mg, with a maximum of 400 mg. Adult Se levels are normally about 81 mg. Many living organisms, such as humans, animals, microbes, and archaea, require the trace element Se as a micronutrient for various vital cellular processes (Novoselov et al. 2002; Rayman 2012; Mangiapane et al. 2014). Additionally, it is necessary for many species’ metabolic processes and growth. Due to its chemical similarity to Sulphur (S), Se can be metabolized via S metabolic pathways. However, Se can serve as a pro-oxidant at high doses and can be hazardous to living organisms because of its ability to substitute S in proteins, which causes the proteins to lose their proper folding. Furthermore, excess Se can cause ROS formation by depleting the intracellular glutathione (GSH) pool (Hoewyk et al. 2008; Grant et al. 2011; Schiavon et al. 2012). Thus, Se nanoparticles have been commonly used in aquaculture feeds as dietary supplements to ameliorate infections and improve disease resistance in aquaculture industries for economically important species like carps and catfish (Tawwab et al. 2007; Han et al. 2011). High resistance to *Aeromonas sobria* infection was found in Nile tilapia fed with dietary Se nanoparticles (1-2 mg/kg) (Ayoub et al. 2021). Another study reported Nile tilapia fishes treated with Se nanoparticles showed increased resistance to *A. hydrophylla* infection (Rathore et al. 2021). *Pangasianodon hypothalamus* exhibited high resistance to *A. veronii* biovar *sobria* when administered dietary Se nanoparticles (1-2 mg/kg) (Kumar and Singh 2019). Additionally, it has been reported that *Labeo rohita* (Hamilton) exposed to 0.3 mg Se nanoparticles/kg demonstrated significantly enhanced resistance to *A. hydrophila* infection (Swain et al. 2019). In this study, synthesis of a novel chitosan-selenium nanoflower (CS-SeNF) was carried out. The bactericidal potential of this CS-SeNF was evaluated against *A. caviae* isolated from contaminated aquaculture water (Fig. 1).

### 2. Materials And Methods

#### 2.1 Synthesis of CS-SeNF

Ascorbic acid was used to reduce sodium selenite in a chitosan solution to synthesize CS-SeNF. Initially 0.01 M Na₂SeO₃ solution was prepared by homogeneously dissolving Na₂SeO₃ in distilled water and a 0.5% Chitosan solution was prepared by dissolving chitosan in 1% acetic acid. The prepared Na₂SeO₃ solutions and chitosan solution was mixed in equal volumes and stirred until homogeneity under dark conditions. Further 0.01 M ascorbic acid solution was added in dark condition under constant stirring at room temperature. Color change from clear to bright orange indicate the reduction of Se salt to Se.
nanoparticles. The solution was centrifuged at 9300 G for 10 min and dried in vacuum oven at 50 °C. The resulting nanoparticle powder was used for further analysis.

2.2 Characterization of CS-SeNF

Different spectroscopic and microscopic methods were used to characterize the synthesized CS-SeNF. Using a UV-vis spectrophotometer, the maximum absorbance of the synthesized CS-SeNF were assessed (UV-1800 Shimadzu, Japan). The CS-SeNF were ground with KBr to obtain the FTIR spectrum, which was then measured with an FTIR spectrophotometer (IR Affinity Shimadzu, Japan). Particle size distribution and zeta potential of the synthesized CS-SeNF were analyzed by dynamic light scattering with particle size analyzer (nanopartica SZ-100V2, HORIBA Japan). Post drop coating the sample onto copper grids, the grids were visualized with a high-resolution transmission electron microscope (120 kV TEM, JEOL Japan). The Bruker D8 Advanced apparatus was used to study the X-ray diffraction pattern. Using a confocal Raman microscope (inViaTM, Reinshaw, UK) equipped with an argon-ion laser at an excitation wavelength of 535 nm, the fabricated CS-SeNF were subjected to spectroscopic Raman examination.

2.3 Bacterial Culture and Maintenance.

Bacteria were isolated from contaminated aquaculture water collected from Mandapam (Latitude: 9° 16’ 48.00” N Longitude: 79° 07’ 12.00” E). The sample was diluted serially and then plated on Zobell Marine Agar (ZMA). A single colony was isolated and plating was done on ZMA by quadrant streaking method and incubated at 25 °C. Further morphological and biochemical identification was done in order to identify the bacteria. DNA was extracted, and 16S rDNA partial sequencing was performed in Barcode Bio Sciences Karnataaka, India. To amplify the 16S rDNA gene using a universal primer, about 50 ng aliquot of the extracted DNA was used. The 16S rDNA sequence was acquired and submitted to the NCBI database.

2.4 Cytotoxicity of CS-SeNF on A. caviae

In order to investigate the cytotoxicity of CS-SeNF, a 3-(4,5-Dimethylthiazol-2-yl) 2,5-Diphenyl tetrazolium bromide dye (MTT) reduction experiment was carried out. The conversion of yellow colored MTT dye to a purple-colored formazan by the mitochondrial dehydrogenase enzyme, which is present in the mitochondria of living cells, serves as a marker for cell viability (Mosmann, 1983). MTT Assay was done to determine the percentage cytotoxicity of the synthesized CS-SeNF on A. caviae. Essentially the bacteria were incubated with various concentrations of the CS-SeNF (0, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL) for 6 h. A volume (200 µL) of CS-SeNF treated cell suspension was taken and about 10 µL of MTT solution was added, and the mixture was then left to incubate for 4 h in the dark. The suspension was centrifuged and washed twice with PBS and the formazan crystals that developed during the procedure were dissolved by addition of 200 µL dimethyl sulfoxide. With the use of a multimode microplate reader (Synergy™ H1, Biotek, USA) the absorbance was recorded at 570 nm. All the experiments were performed in triplicates, the data presented are means ± s.d.

2.5 Lipid Peroxidation in A. caviae by exposure to CS-SeNF
Following the method of Sayeed et al., Malondialdehyde (MDA), a significant indicator of lipid peroxidation was quantified. A. caviae bacteria were exposed to various CS-SeNF concentrations (Sayeed et al. 2003). Briefly, 200 µL of bacterial cells treated with SeNPs were added to 700 µL of HCl (0.1 M), and the mixture was then let to sit at room temperature for 20 min. Additionally, 900 µL of thiobarbituric acid (TBA) (0.025 M) was added and the solution was allowed to stand for 65 min at 37 °C. Post incubation, 400 µL of 10 mM PBS was added, and using a hybrid multimode microplate reader (Synergy™ H1 Biotek, US), the fluorescence was measured at 520 nm (excitation) and 549 nm (emission). The percentage of lipid peroxidation by calculating MDA values and the data was expressed in terms of concentration of CS-SeNF (in µg/mL) vs percentage of lipid peroxidation.

2.6 Estimation of ROS production by DCFDA Assay

Spectrophotometric analysis of ROS production was performed using the fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA). The non-fluorescent chemical 2',7'-dichlorofluorescein is produced when cellular esterase hydrolyses DCFH-DA, which then diffuses easily through the cell membrane (Wang and James 1999). It rapidly oxidises to extremely fluorescent 2',7'-dichlorofluorescein in the presence of ROS. The bacterial cells of A. caviae were exposed to various concentrations of CS-SeNF (0, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL). Briefly the bacterial culture treated with different concentrations of CS-SeNF were incubated in dark conditions with of DCFH-DA (200 µM). The multimode hybrid microplate reader (Synergy™ H1 Biotek, US) was used to detect the fluorescence. A maximum excitation of 490 nm was applied and the emission was recorded at 523 nm.

3. Results And Discussion

3.1 Synthesis and characterization of CS-SeNF.

The visual observation of change in colour from colourless to bright orange indicated the complete reduction and formation of the CS-SeNF. The UV-vis spectrum (Fig 2A) revealed an absorbance maximum at 265 nm which is characteristic of the reduction of selenium salt to CS-SeNF. According to published research, the absorbance maxima of Se nanoparticles fabricated with reducing agents including carboxylic groups are predicted to be in the 200-300 nm range (Tran et al. 2016). In the FTIR Spectrum (Fig 2B), peaks at 1648 cm\(^{-1}\) and 1655 cm\(^{-1}\) correspond to asymmetrical and symmetric stretching vibrations of COO\(^-\). The peak obtained at 1378 cm\(^{-1}\) indicates the symmetric stretch of COO\(^-\) in chitosan. The shift in peak to 1415 cm\(^{-1}\) indicated the involvement of COO\(^-\) groups in the synthesis of CS-SeNF. The band at and 3420 cm\(^{-1}\) corresponds to the O-H stretching. The IR band obtained at 2849 cm\(^{-1}\), is indicative of the presence of Aliphatic C-H vibrations. The band at 890 cm\(^{-1}\) correspond to =C-H out of plane bending and -(CH\(_2\)\(_n\))\(_n\). It was found that the prepared CS-SeNF were polydisperse in nature and found to have a unique and novel nanoower morphology. Particle size measurement (Fig 3A) resulted in average size of particle with 186.3 nm. Zeta potential (Fig 3B) of the synthesised CS-SeNF was found to be 49.1 mV which indicated significantly stable colloidal nanoparticles. Electrostatic and electro-steric forces prevent particles from sticking to other components in the medium, such as organic compounds,
therefore their stability and capacity to aggregate largely depend on their surface charge (Dutta et al. 2011). The presence of hydrophobic groups on the surface can successfully reduce the toxicity to considerable levels with the usage of amphiphile coatings. (Simoes et al. 2021).

The transmission electron microscopic imaging of the synthesized CS-SeNF revealed unique nanoflower like morphology (Fig 4). XRD analysis was used to examine the phase of the synthesised CS-SeNF. The XRD pattern obtained of the synthesised CS-SeNF seen in (Fig 5A) clearly revealed that the nanoparticles were amorphous in nature. Using Raman spectroscopic technique, nature, and bonding nature of synthesized CS-SeNF were assessed. The Raman scattering spectra acquired for CS-SeNF (Fig 5B) showed a sharp resonance peak at 254 cm$^{-1}$ was a characteristic absorption band for monoclinic Se and α-Se (Lucovsky et al. 1967). The obtained resonance peak is indicative of Se$_8$ absorption band in the amorphous material (Baganinch et al. 1991).

3.2 Bacterial Isolation and Identification

Bacteria were isolated by serial dilution from contaminated aquaculture water. The isolated bacteria were subjected to morphological and biochemical characterizations. In addition to fundamental biochemical tests, PCR-based molecular methods that amplify conserved sections of the 16S rDNA gene have also shown promise for phylogenetic discrimination. Small amounts of DNA isolated from laboratory cultures or natural habitats can be used in PCR to recover the 16S rDNA gene sequences, which can be used to characterise the strain. These sequences are independent of cultivation or growth conditions (Giovannoni 1991). Bacterial relationships can be determined since the 16S rDNA gene is present in every bacterial species. In general, identifying strains at various levels, including what we currently refer to as the species and subspecies level, is made possible by comparing the 16S rDNA gene sequences, which allows discrimination among organisms at the genus level across all major phyla of bacteria. The 16S rDNA partial sequence was acquired and submitted to the NCBI database. with an accession number (ON203034). A nucleotide BLAST analysis was performed to find potential related sequences by comparing them to non-redundant BLAST databases. The results clearly indicated similarity with *Aeromonas caviae*. The bacteria were identified as *Aeromonas caviae* after comparison with the 16S rDNA sequence data from strains available at EMBL, GenBank and DDBJ. A Phylogenetic tree was constructed using closely related and highly similar sequences to arrive at the evolutionary relationship using the Mega X software (Fig 6).

3.2 Cytotoxicity of CS-SeNF on *A. caviae*

Cell viability is determined by mitochondrial dehydrogenase, an enzyme found in the mitochondria of living cells, which reduces MTT to purple-coloured formazan. (Mosmann, 1983). The cytotoxicity was found to increase in dose-dependent manner. The maximum cytotoxicity of 75.06 % was observed at 1000 µg/mL. At the lowest concentration 15.625 µg/mL a cytotoxicity of 28.09 % was observed which was significantly higher than untreated *A. caviae* (Fig 7). This indicates that the synthesised CS-SeNF have significant cytotoxicity against pathogenic *A. caviae*.
3.3 Lipid Peroxidation in *A. caviae* on exposure to CS-SeNF

Malondialdehyde (MDA), the result of lipid peroxidation based on interaction with thiobarbituric acid, was quantified to determine the degree of lipid peroxidation of the CS-SeNF (Sayeed et al. 2003). The induction of lipid peroxidation process in an organism is attributed to the presence of free radicals. Lipid peroxidation is a sign of oxidative stress in living organisms as it damages the lipids in cell membranes. As a result, it serves as a biomarker for oxidative stress. The percentage of lipid peroxidation was assessed by the amount of MDA. The Lipid peroxidation was found to increase with an increase in dose (Fig 8A). This indicated the production of ROS induced damage in the bacteria. Increased MDA synthesis could result in the development of mutagenic substances that can interact with DNA to create various DNA addition products, including deoxyguanosine adducts (Saieva et al. 2016).

3.4 ROS Production in *A. caviae*

Using the DCFH-DA test on *A. caviae*, the contribution of SeNPs to ROS induction has been shown. The intensity of fluorescence of 2',7'-dichlorofluorescein determines the quantity of ROS produced. All live cells generate ROS as a by-product of metabolism. Bacteria produce elevated ROS in stress condition. Superoxide dismutase and other ROS scavenging enzymes found in bacteria work to reduce the ROS produced under non-stressful conditions (Arakha et al. 2015). Due to the significant involvement of reduced oxygen intermediates in signal transduction and the production of transcription factors, these ROS are essential for cellular function (Martindale and Holbrook 2002). Excess ROS interacts with proteins, lipids, and DNA to disrupt or destroy their intended functions, which can cause significant damage. ROS production in CS-SeNF treated *A. caviae* was concentration dependant (Fig. 8B). Increase in the concentration of CS-SeNF resulted in significant increase in the fluorescence intensity. The increase in ROS generation is directly associated with the change in fluorescence intensity. As far as biological processes such as the stress response is concerned, ROS serve as secondary messengers. ROS is exceedingly harmful at high quantities and triggers oxidative damage. The inactivation or modification of biomolecules by ROS can lead to the cellular dysfunction, alterations in cellular structure, and mutagenesis (Rezayian et al. 2019). The treatment and exposure of *A. caviae* with increasing concentrations of the CS-SeNF induced the ROS production. At higher concentrations the ROS damages the cellular function by degradation of lipids proteins and causes cellular dysfunction. Thus, the antimicrobial activity of CS-SeNF could be attributed to cell damage due to excessive ROS production.

**Conclusion**

In summary, the toxicity of CS-SeNF on pathogenic *A. caviae* strain was found to be significant as demonstrated by *in vitro* assays CS-SeNF can be used as an antimicrobial agent to inhibit the growth of *A. caviae* and can potentially be used in aquaculture industries. Similar toxicity experiments were performed on food pathogen *Staphylococcus aureus* and it was demonstrated that Se nanoparticles have significant inhibitory effects. Significant inhibitory effects of selenium nanoparticles were observed on *S. aureus* at concentrations of 20-50 µg/mL (Nguyen et al. 2017). However, more research is necessary to
completely comprehend the mechanisms of the cytotoxicity and antibacterial properties of CS-SeNF and
to investigate into their potential application.

Declarations

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Ethical Approval

Not Applicable

Competing interests

The authors affirm that they have no known financial or interpersonal conflicts that would have appeared
to have an impact on the research presented in this study.

Authors' contributions

Prasad Sowmiya-Experimental Analysis, Conceptualization, Writing Original Draft, Review and Editing;
Tharmathass Stalin Dhas-Conceptualization, Visualization, Supervision, Review and Editing;
Dhinakarasamy Inbakandan-Review and Editing; Ravi Mani-Review and Editing, Anandakumar Natarajan-
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Availability of data and materials

The authors approved the availability of data and materials for publishing the manuscript.

References


**Figures**

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